include, but are not limited to, chemical treatment, using chemical agents such as N-methyl-N'-nitro-N-nitrosoguanidine, nitrosourea, nitrogen mastard, etc., and physicochemical treatments, such as ultraviolet light irradiation, radiation exposure with cobalt-60 or the like, heating, etc. Culture of artificial mutant strains, purification of toxins from the mutant strains, and verification of the activity thereof are carried out essentially in the same manner as for the wild-type strains.

A recombinant mutant toxin can be produced by recombinant cells. Methods for the production of such cells are conventional in the art. The following is an illustrative example of a method for producing a recombinant heat-labile toxin mutant of *E. coli*. A toxin-overproducing recombinant strain is prepared from a strain producing heat-labile toxin separated from patients (*Escherichia coli* 1032 strain), for example, by using the method described by Tamura et al. (S. Tamura et al., Vaccine 12, 1083-1089, 1994). As the first step, separation treatment is carried out to obtain a plasmid (65 kbp) containing both genes encoding heat-labile toxin and thermostable toxin. The plasmid is treated with restriction enzymes and such to cut out the heat-labile toxin gene (6.7 kbp). The resulting fragment is ligated into a plasmid, such as pBR322 or the like. The fragment is amplified by PCR or the like. The heat-labile toxin genes are then ligated into other expression vectors. Subsequently, site-specific mutagenesis is performed on the vector, by the method described by Ho et al. (S. N. Ho et al., Gene 77, 51-59, 1989) for example. The fragment of interest is then amplified by PCR method, using a pair of selection primers in order to give a recombinant *E. coli* strain producing recombinant mutant toxin. In the next step, the bacterial strain is cultured according to the method described by Clements et al. (J.D. Clements et al., Infect. Immun. 24, 760-769, 1979), for example. The protein of interest is purified from the culture supernatant by ultrafiltration, ammonium sulfate precipitation, agarose gel chromatography (elution with a buffer containing galactose) and others. It is thus possible to obtain recombinant mutant toxins derived from the heat-labile toxin of *E. coli* that is infectious to human. Alternatively, natural toxins can be obtained when the

site-specific mutagenesis is not conducted in the process described above.

It is possible to replace amino acid residues with other residues at desired positions in the amino acid sequence of a toxin, for example, by using site-specific mutagenesis as described above. On the other hand, specific amino acid residues, for example a glutamic acid residue, may remain unchanged at particular positions. Sugar residues can also be treated basically in the same manner. The mutants obtained are screened to select those having adjuvant activity, which selection yields the mutant toxins of the invention. Each mutant may exhibit the toxic activity at any level. In other words, when the adjuvant activity is satisfactorily maintained but the level of toxic activity of the mutant is comparable to that of the natural one, then the mutant toxin can be used as the adjuvant after proper attenuation treatment as used for the natural one. Otherwise, if the adjuvant activity is satisfactorily maintained and the level of toxic activity of the mutant is preferably low, then the mutant toxin can be used as the adjuvant of attenuated toxin without any modification or after subjecting to some attenuation treatment. Although trial-and-error experimental approaches are required to obtain the mutants, such approaches generally have the advantage that the resulting recombinant mutant toxins hardly revert to the original toxic activity.

Method for attenuating toxin:

Before using the adjuvants of the invention, one must first verify whether or not the toxins produced by the wild-strain, artificial mutant strain, or recombinant mutant strain have the activity of enhancing immunity after being attenuated. The toxins can be attenuated by any conventional method. Examples of suitable attenuation methods are described below. The term "attenuation" means herein that the toxic activity is reduced by using any means and it should not be construed as being limited to the examples. However, these methods are convenient and reliable to provide the effect of treatment.

All of these attenuation methods are publicly known methods used for converting to toxoids. In the present invention, however, it is

required to attain high levels of attenuation in which toxic activity is reduced to one-two thousandth. Accordingly, more extreme conditions are required for the attenuation in the present invention, although the methods to be used here are based on the same principles as the publicly known methods. Specifically, for example when cholera toxin is treated with formalin, temperature for the treatment is 5 to 30°C (ordinary 5°C), the concentration of formalin is 0.5 to 0.8% (ordinary 0.3%), and the duration of treatment is 20 to 70 days (ordinary about 7-14 days), or the like.

[1] Chemical method

The toxin is dissolved at an adequate concentration in a buffer. While the pH is adjusted to be within a range where the toxin is stable (typically, pH 5 to 8), an agent, for example, formalin, glutaraldehyde, phenol, iodine, acid anhydride, or a detergent such as bile acid with the adequate concentration (typically, 0.01 to 1.0%) is added stepwise. Then the solution is incubated at an adequate temperature (for example, 5°C). After incubation, the agent is removed by any suitable method such as dialysis. The residual toxic activity is then assayed. If desired, the treated toxin is incubated again at a temperature at which the attenuated toxin will be used practically, for example, at 37°C, in order to secure that the toxic activity does not recover. The attenuated toxin of interest is thus yielded. This method is based upon the previous method widely used for the toxoid conversion, and is also suitable for large-scale production. However, it is preferable to use a method with which the toxic activity does not recover. There are some conventional methods used for this purpose. Exemplary methods include but are not limited to (1) the simultaneous addition of lysine in a quantity corresponding to the concentration of the agent for the attenuation treatment, for example, formalin, and (2) the performance of a reduction treatment ¥ after the attenuation.

[2] Physical method

The toxin is dissolved at an adequate concentration in a buffer. While the pH is adjusted to a pH at which toxic activity is generally lost, including an acidic pH (for example, pH 2 to 4) or alkaline